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PATENT

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Applicant: Weigel et al)	Atty Dkt No: 5820.603
)	
Serial No: 09/842,930)	Examiner: L. Spector
)	
Filed: April 25, 2001)	Art Unit: 1647
)	
For: HYALURONAN RECEPTOR)	
FOR ENDOCYTOSIS)	
(as amended))	

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Commissioner for Patents
Washington, D.C. 20231

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DECLARATION OF PAUL H. WEIGEL, Ph.D. UNDER 37 C.F.R. § 1.131

Sir:

I, Paul H. Weigel, of lawful age declare:

1. I am an Applicant of the above-referenced patent application and a co-inventor of the invention claimed in the subject application.
2. I have reviewed the reference by Zhou et al., Journal of Biological Chemistry (1999) 274:33831, which was cited by the Examiner in the rejection under 35 USC § 102(a)/103(a) made in the Official Action mailed August 16, 2002 in the present application, and which lists Bin Zhou, Janet A. Oka, Anil Singh, and Paul H. Weigel as authors.

3. Submitted herewith as attachment A is a copy of the original manuscript submitted to the Journal of Biological Chemistry on July 23, 1999 which subsequently matured into the Zhou et al. publication. Submitted herewith as attachment B is a copy of the Cover Letter to the Editor, received via email from the Journal of Biological Chemistry on August 24, 1999. Submitted herewith as attachment C is a copy of the reviewer's comments received via email from the Journal of Biological Chemistry on August 24, 1999.

4. Attachment A demonstrates that the inventive concept recited in the claims of the subject application were conceived and constructively reduced to practice at least as early as the July 23, 1999 submission date. The differences between Attachment A and the Journal of Biological Chemistry paper subsequently published are described herein below.

The original submission (attachment A) contained 5 figures. In response to a reviewer's request for more information about the specificity of the monoclonal antibody used to purify the HA receptor, data that further exemplified and clarified the information originally submitted was added as a new Figure 1. The previous 5 figures were unchanged, but were then renumbered (as Figs 2-6) in the revised manuscript, which was submitted on August 30, 1999.

The fact that the revised manuscript was returned to the Journal in less than one week confirms that no new research was performed and that no "new"

data was obtained; minor revisions to the text were made to incorporate data that further exemplified and clarified the information originally submitted concerning the specificity of the HARE monoclonal antibody, and the figures were renumbered. In addition, the period between August 24 and August 30 was also used to create TIFF files for electronic submission of the figures and the revised manuscript, since the Journal was making the transition at that time to electronic submission of paper. This was our first experience with having to create high quality files for electronic submission, and therefore this was the most time-consuming task of the period between August 24 and August 30, 1999.

Further evidence that the inventive concept recited in the claims of the subject application were conceived and constructively reduced to practice at least as early as the July 23, 1999 submission date can be found in the cover letter to the Editor (attachment C), which specifically describes the minor changes made to the text, including shortening the paper as requested. In addition, page 2 of Attachment C is a summary of results, for the reviewer's information only, that we included as a supplement (page 2) to the cover letter to the Editor. This supplemental data page should also serve to confirm that the inventive concept recited in the claims of the subject application were conceived and constructively reduced to practice at least as early as the July 23, 1999 submission date.

5. Submitted herewith as Attachment D are copies of correspondence and a manuscript submitted to Biochemistry on April 28, 1999. This manuscript, which was submitted almost three months prior to the Journal of Biological Chemistry submission, was virtually the same manuscript, except that one more figure was included. This earlier manuscript had 6 figures; numbers 2-6 were identical to figures 1-5 in the original Journal of Biological Chemistry manuscript (which were figures 2-6 in the final accepted and published paper). The first figure in the Biochemistry submission showed the ability of a variety of different plant lectins to bind to the HARE protein. Since these data were not critical to the documentation of how HARE was purified as well as the subunit characterization of the proteins, it was not included in the Journal of Biological Chemistry communication, which has a strict page (length) limit. The reviews for the manuscript submitted to Biochemistry (dated July 7, 1999) were split. One reviewer considered the results too preliminary and not appropriate for the journal, whereas the second reviewer was enthusiastic and said "The experiments presented are well performed and in my opinion this work should definitely be published." The Editor in these situations usually takes the view of the more critical reviewer. Thus the manuscript was not accepted by Biochemistry, and we then reorganized it slightly and submitted it to the Journal of Biological Chemistry. Virtually the same data and manuscript were then accepted by the Journal of Biological Chemistry as a rapid communication -

denoting an important and timely study deserving of accelerated publication.

Therefore, Attachment D actually serves to establish conception and constructive reduction to practice of the inventive concept recited in the claims of the subject application at least as early as April 28, 1999.

6. In summary, Attachments A-C demonstrate that the invention as currently claimed in the subject application was conceived and constructively reduced to practice at the very least as early as July 23, 1999, while Attachment D demonstrates that the invention as currently claimed in the subject application was conceived and constructively reduced to practice at the very least as early as April 28, 1999.

6. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

10/2/03
Date

Paul H. Weigel
Paul H. Weigel, Ph.D.



ATTACHMENT "A"

submitted: July 23, 1999

COMMUNICATION

PURIFICATION AND SUBUNIT CHARACTERIZATION OF THE RAT LIVER

ENDOCYTIC HYALURONAN RECEPTOR

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Running Title: Purification of the LEC endocytic HA receptor

Key words: hyaluronic acid, hyaluronan binding protein, receptor mediated endocytosis, recycling receptor, iso-receptors

ABSTRACT

The endocytic hyaluronan (HA) receptor of liver sinusoidal endothelial cells (LECs) is responsible for the clearance of HA and other glycosaminoglycans from the circulation in mammals. We report here for the first time the purification of this liver HA receptor. Using lectin and immuno-affinity chromatography, two HA receptor species were purified from detergent-solubilized membranes prepared from purified rat LECs. In nonreducing SDS-PAGE, these two proteins migrated at 175 kDa and ~ 300 kDa corresponding to the two species previously identified by photoaffinity labeling of live cells as the HA receptor (Yannariello-Brown, et al. *J. Biol. Chem.* **267**, 20451, 1992). These two proteins co-purify in a molar ratio of 2:1 (175:300) and both proteins are active, able to bind HA after SDS-PAGE, electrotransfer and renaturation. After reduction, the 175 kDa protein migrates as a ~185 kDa protein and is not able to bind HA. The 300 kDa HA receptor is a complex of three disulfide-bonded subunits that migrate in reducing SDS-PAGE at ~ 260, 230 and 97 kDa. These proteins designated, respectively, the α , β , and γ subunits are present in a molar ratio of 1:1:1 and are also unable to bind HA when reduced. The 175 kDa protein and all three subunits of the 300 kDa species contain N-linked oligosaccharides, as indicated by increased migration in SDS-PAGE after treatment with N-glycosidase-F. Both of the deglycosylated, nonreduced HA receptor proteins still bind HA.

HA¹ is an important component of the extracellular matrix of all tissues and plays an important role in development, cell proliferation, cell adhesion, recognition, morphogenesis, differentiation and inflammation (1-4). The daily total body turnover of HA in humans is estimated to be at least 1 g/day (4). HA degradation and removal in the body occurs via two clearance systems (3): one is in the lymphatic system, which accounts for ~85% of the HA turnover, and another system is hepatic, accounting for ~15% of the total body HA turnover. HA in the extracellular matrices of tissues throughout the body is continuously synthesized and degraded. Very large HA molecules ($\sim 10^7$ Da) are partially degraded to large fragments ($\sim 10^6$ Da) that are then released from the matrix and flow with the lymph to lymph nodes. The majority of HA (~85%) is completely degraded in the lymph nodes by unknown cell types and unknown mechanisms and the remaining HA (~15%) that passes through the nodes finally enters the blood. Clearance of this circulating HA is presumably important for normal health (3,4). Elevated serum HA levels are found in several disease conditions such as liver cirrhosis, rheumatoid arthritis, psoriasis, scleroderma and some cancers (5-7).

The liver sinusoidal endothelial cells (LECs) have a very active recycling, endocytic receptor that removes HA and other glycosaminoglycans, such as chondroitin sulfate, from the circulation (3,8-10). Earlier reports misidentified this LEC HA receptor as ICAM-1 (11,12), also known as CD54, which is a 90 kDa protein. This finding was later recognized as an artifact in that ICAM-1 bound nonspecifically to the HA affinity resin employed in the putative purification (13). In two previous studies, one using a photoaffinity derivative of HA (14) and the other using a novel ligand blot assay with ¹²⁵I-HA (15), we identified two specific HA-binding proteins in isolated rat LECs at 175 kDa and ~300 kDa. In the present study, we have finally purified these two proteins for the first time. Our results show the ~300 kDa HA receptor protein contains three subunits after reduction but does not contain the 175 kDa HA

receptor protein, which itself contains no other subunits.

EXPERIMENTAL PROCEDURES

Materials RCA-I-agarose gel was purchased from EY laboratories, Inc. Tris, SDS, ammonium persulfate, N, N'-methylenebisacrylamide, and SDS-PAGE molecular weight standards were from Bio-Rad. Na¹²⁵I was from Amersham Corp. NP-40 was from CalBiochem. HA (human umbilical cord) from Sigma, was purified as described previously (16). Nitrocellulose membranes were from Schleicher & Schuell. Acrylamide and urea were from U.S. Biochemical Corp. p-Nitrophenylphosphate was from Kirkegaard & Perry Laboratories. N-glycosidase F (EC 3.5.1.52) and all other chemicals, which were reagent grade, were from Sigma. TBS contains 20 mM Tris-HCl, pH 7.0, 150 mM NaCl.

Preparation of LECs and LEC membranes—Male Sprague Dawley rats were from Harlan, Indianapolis, IN. LECs were isolated by a modified collagenase perfusion procedure (17), followed by differential centrifugation and then discontinuous Percoll gradient fractionation. Cells were collected from the 25/50% interface and washed 3-times with PBS at 4°C. For preparation of LEC membranes, the cells were hypotonically swollen, homogenized, and centrifuged at 1000xg. The supernatant was then centrifuged at 105,000 xg to obtain the total membrane fraction (18).

Ligand blot assay. LECs, membranes or protein samples were solubilized in an SDS sample buffer containing 16 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% glycerol (v/v), and 0.01% bromophenol blue (19). No reducing agent was added unless as noted in the figures. Cell or membrane samples were sonicated on ice for 10-20 s. After SDS-PAGE, the contents of the gel were electrotransferred onto a 0.1 µm nitrocellulose membrane for 2 h at 24 V at 4°C using 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol and 0.01% SDS. The nitrocellulose was treated with TBS containing 0.05% Tween-20 at 4°C for 2 h or overnight and then incubated with 2 µg/ml ¹²⁵I-HA in TBS without or with a 150-fold excess of HA (as competitor) to assess total and

nonspecific binding, respectively. The nitrocellulose membrane was washed 5-times for 5 min each with 0.05% Tween-20 in TBS and dried at room temperature. The ^{125}I -HA bound to protein was detected by autoradiography with Kodak BioMax film. Nonspecific binding in this assay is typically <5% (15).

Purification of the HA receptor. Total LEC membranes from 18 rats were suspended in 200 μl TBS containing 2% NP-40 (v/v) and gently mixed by rotation at 4°C for 2 h. The solubilized membranes were then diluted with TBS to a final NP-40 concentration of 0.5% and centrifuged for 30 min at 100,000xg. The supernatant extract was then loaded at room temperature onto a RCA-I gel column containing 10 ml of resin. RCA-I lectin was chosen because HAR binding was quantitative, easily displaced with lactose and a significant purification from other membrane proteins was obtained. The column was washed with 10 volumes of TBS containing 0.05% Tween 20. The bound proteins were eluted with 100 mM lactose in distilled water. The eluate was dialysed (using dialysis tubing) against multiple changes of TBS at 4°C overnight, concentrated about 10-fold using a Centricon-30 device (from Amicon), and then passed slowly over an immuno-affinity column (~8 ml), containing monoclonal antibody 175HAR-30 coupled to CNBr-activated Sepharose (~2 mg/ml resin). Details on the preparation and characterization of this and other monoclonal antibodies raised against the rat LEC 175 kDa HAR will be described elsewhere². The affinity column was washed with 10 volumes of 0.05% Tween-20 in TBS and then eluted with 100 mM sodium citrate, pH 3.0. Eluted fractions were immediately neutralized by collection into 1 M Tris. The eluted fractions containing protein were pooled, dialysed against TBS at 4°C overnight and then concentrated using a Centricon-30.

Deglycosylation of HA receptors with N-glycopeptidase F. Purified HA receptor (1.17 μg) was heated with 0.5% SDS at 90°C for 3 min. Samples (22 μl) were chilled on ice for 4 min, and then 0.5 M Tris-HCl, pH 7.2 was added to a final concentration of 10 mM. One-half unit of N-

glycopeptidase F (20) and distilled water were added to give a final volume of 25 μ l. The samples were incubated at 37°C overnight, 9 μ l of 4-fold concentrated SDS sample buffer was added and they were heated for 3 min at 90°C. The samples were subjected to SDS-PAGE, and protein was detected by silver staining or receptor activity was determined by the 125 I-HA ligand blot assay.

Two-dimensional electrophoresis. Affinity-purified HA receptor (~1.5 μ g) was subjected to SDS-PAGE without reduction and the gel was stained with Coomassie blue. The 175 kDa and 300 kDa proteins were excised from the gel, cut into smaller pieces, divided into two portions and incubated at 90°C for 4 min with SDS sample buffer with or without 10 mM DTT followed by 50 mM iodoacetamide. The samples were then subjected to a second dimension of SDS-PAGE without reducing agent. Proteins were visualized by silver staining (21) and HA receptor activity was assessed by the 125 I-HA ligand-blot assay.

General. Protein content was determined by the method of Bradford (22) using BSA as a standard. Receptor protein content was assessed after precipitation with 5% trichloroacetic acid to remove detergent. SDS-PAGE was performed according to the method of Laemmli (19). 125 I-HA was prepared using Iodogen (Pierce) and a uniquely modified hexylamine derivative of HA, which was synthesized and radiolabeled as described previously (16). 125 I radioactivity was measured using a Packard Auto-Gamma Counting system.

RESULTS AND DISCUSSION

In addition to the normal turnover of HA in tissues throughout the body, a wide range of biomedical and clinical applications use exogenous HA that is also removed from the lymphatics or ultimately from the blood and degraded by the LEC HA receptor (3,4). For example, HA is used extensively in eye surgery (23), in the treatment of joint diseases including osteoarthritis (24) and is being developed as a drug delivery vehicle (25). Numerous studies

have explored the benefit of HA during wound healing (26,27). The exogenous HA introduced in these various applications is naturally degraded by the lymph and LEC systems noted above. Despite the very large endocytic and degradative capacity of the LEC HAR (28) and its importance in removing HA from the blood, the HA receptor had not yet been successfully purified.

A breakthrough in the ability to purify the LEC HAR occurred with our discovery that two very active and specific HA-binding proteins could be detected in LECs by ligand blotting using ^{125}I -HA (15). The 175 kDa and 300 kDa HA-binding species in LECs are readily detected after renaturation with Tween 20. The HA-binding activity at 175 kDa and 300 kDa in ligand blots corresponds perfectly to our previous identification of two HAR proteins on intact LECs by the use of an HA photoaffinity derivative. This HA probe specifically photo-labeled proteins of 175 kDa and 300 kDa (14). The two HAR species observed by ligand blotting with ^{125}I -HA also showed the same specificity with a panel of polyanionic competitors (15) as observed for intact LECs (9,10).

Sequential lectin and immuno-affinity chromatography purifies the two LEC HAR species to homogeneity. We have prepared several useful monoclonal antibodies against the rat LEC 175 kDa HAR that recognize this protein in Western blots after either nonreducing and/or reducing SDS-PAGE². The antibody 175HAR-30 was able to bind the 175 kDa HAR species, and also recognize the 300 kDa HAR species. However, as described below, the 300 kDa species is not a dimer of the 175 kDa protein and does not contain a 175 kDa subunit. This 175HAR-30 antibody was also able to immunoprecipitate both HAR proteins from LEC extracts, thus enabling the 175 kDa HAR and the 300 kDa HAR proteins to be purified for the first time (Fig. 1). Nonreducing SDS-PAGE analysis showed that these two proteins comprise >98% of the final purified HAR preparations. Based on silver and Coomassie blue staining of nonreduced gels, the 175 kDa HAR and 300 kDa HAR proteins are purified in an apparent

molar ratio of 2:1. The fraction of total staining in the 175 kDa band was 0.47 ± 0.07 (n=5) and 0.55 ± 0.09 (n=3), respectively, for Coomassie and silver stained gels.

The two purified HAR proteins remained active, as assessed by the ligand blot assay (Fig. 2, lane1). Both the 175 kDa and ~300 kDa HAR proteins were shifted to a lower mass by treatment with N-glycosidase F, indicating that both HAR species contain N-linked oligosaccharides (Fig. 3, lanes 1 and 2). The de-N-glycosylated 175 kDa HAR and 300 kDa HA receptors were still capable of 125 I-HA binding (Fig. 2, lane 2). Therefore, N-linked oligosaccharides do not appear necessary for the HA-binding activity of these receptors. However, the reduced 175 kDa and 300 kDa HAR proteins no longer bind 125 I-HA (Fig. 2, lane 3).

Subunit composition of the two HAR proteins. To determine if either protein contains disulfide-bonded subunits, the copurified 175 kDa HAR and 300 kDa HAR were analyzed by reducing SDS-PAGE (Fig. 3, lanes 3 and 4). After reduction with β -mercaptoethanol, four protein species were evident ranging in apparent size from 97 kDa to 260 kDa. In order to determine which HAR species yielded each of these four proteins, the 175 kDa HAR and 300 kDa HAR were first separated by nonreducing SDS-PAGE. The two HAR bands were then excised, and reanalyzed by SDS-PAGE with or without reduction with β -mercaptoethanol (Fig. 4). The reduced 175 kDa HAR yielded no other protein species, but the apparent size of the protein increased to ~185 kDa (Fig. 4, lane 3). This shift to higher M_r is typical of membrane receptors with extracellular domains whose compact or tightly folded structures require intraprotein disulfide bridges.

After reduction, the 300 kDa HAR gave rise to three protein species with apparent masses of 260, 230 and 97 kDa, which we designate, respectively, as the α , β , and γ subunits of the 300 kDa HAR (Fig. 4 lane 2). None of these three reduced proteins were able to bind 125 I-HA (Fig. 2, lane 3). All three subunits contain N-linked oligosaccharides (Fig. 3, lane 4). Based on

Coomassie blue and silver staining, and their apparent sizes, the molar ratio of the three protein components of the 300 kDa HAR is 1:1:1 (Table I). The 300 HAR could be a ($\alpha\beta\gamma$) heterotrimer with these three subunits being the products of several different genes. Alternatively, the 300 HAR could be a homodimer of ~300 kDa subunits, with one subunit specifically cleaved into ~97 and ~230 kDa species. In purified HAR preparations, the stoichiometry of the 175 kDa and 300 kDa proteins has consistently been 2-3:1 (175:300). Therefore, the overall stoichiometry of the four proteins in reduced, affinity purified HAR preparations was 2:1:1:1, respectively, for the 175 kDa protein and the α , β , and γ subunits of the 300 kDa complex (Fig. 5).

Although the 175 and 300 kDa species could represent monomeric and dimeric forms, respectively, of the LEC HA receptor, based on the present results, we find this is not the case. The 175 kDa HAR is not a covalently bound part of the 300 kDa HAR complex. Nonetheless, our earlier photoaffinity approach (14) identified the correct HAR proteins. Likewise, the ligand blot assay, subsequently developed to identify the LEC HAR (15), also monitors the same proteins that were purified in the present study. Our results, therefore, have consistently identified proteins of 175 kDa and ~ 300 kDa as the LEC HAR.

These points are relevant in light of earlier reports that the LEC HA receptor was not larger than 100 kDa (11) and was, in fact, ICAM-1 (12). ICAM-1, which is also designated in lymphocytes as CD54, is not a likely candidate for the very active endocytic HA receptor of LECs because ICAM-1 is not a recycling receptor that operates via the coated pit pathway (29).

Although this identification of the LEC HAR as ICAM-1 is now recognized to be incorrect and was withdrawn (13), erroneous studies based on this report were published (30,31) and may be widely cited.

A 1:1:1 complex of the three 300 kDa subunits might be expected to migrate as a >500 kDa species in nonreducing SDS-PAGE. Although the lack of good standards above 200 kDa

makes it difficult to assign relative mass, the 300 kDa HAR appears to migrate anomalously fast (*i.e.* to a smaller than appropriate size position). We have noted before (15) that the 175 kDa HAR and 300 kDa HAR are very elongated, not globular, molecules. Their apparent M_r s depend greatly on the pore size of the gels. During SDS-PAGE, the 175 kDa and 300 kDa HAR proteins behave like elongated rods (15). Ferguson plot analysis showed that their apparent size relative to the typical globular standards was very dependent on the pore size of the gels. Therefore, the anomalous migration of the nonreduced 300 kDa HAR may be explained if the two large α and β subunits are also very extended or rod-like in the ternary complex (Fig. 5).

We propose that LECs contain the 175 kDa and 300 kDa species as two highly similar but distinct and separate isoreceptors for HA. The consistent 2:1 stoichiometry of the purified 175 kDa HAR and 300 kDa HAR species in LECs may reflect the tight and coordinated regulation of their expression, rather than their physical association. The reason for having two HARs may be related to the great polydispersity of HA. More than one HAR may be required to mediate effective removal from the blood of HA molecules that can vary over a mass range from 10^3 to 10^6 Da. Each HA isoreceptor may be specialized to interact with either smaller or larger HA. Ongoing studies to clone and further characterize the four purified HAR subunit proteins will enable us to determine their primary structures and their roles in normal health and a variety of diseases.

ACKNOWLEDGEMENTS

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FOOTNOTES

1. Abbreviations. HA, hyaluronic acid, hyaluronate, hyaluronan; HAR, HA receptor; ICAM-1, intercellular adhesion molecule-1; LECs, liver endothelial cells; Tris, trishydroxymethylamino methane; TBS, tris-buffered saline.
2. B. Zhou, J.A. Oka and P. H. Weigel (manuscript in preparation).

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FIGURE LEGENDS

Figure 1. Immuno-affinity purification of the LEC HA Receptors. HA receptors were purified from NP-40 extracts of LEC membranes as described in Experimental Procedures. Protein profiles were analyzed by SDS-PAGE and silver staining. Lane 1, run-through from the RCA-I column; lane 2, the starting NP40 extract of LEC membranes; lane 3, proteins purified from the RCA-I column; lane 4, the purified HA receptors eluted from the antibody column. The solid and open arrows indicate the ~300 kDa and 175 kDa positions, respectively.

Figure 2. Effects of reduction and N-glycosidase F treatment on HA-binding activity of the purified 175 kDa HAR and 300 kDa HA receptors. Purified LEC HAR was reduced and/or N-glycosidase F-treated and assayed after SDS-PAGE by ligand blotting with ^{125}I -HA. Both the 175 kDa (open arrow) and the 300 kDa (solid arrow) HAR species are smaller after deglycosylation by 25-30 kDa but both species are still active (lanes 1 & 2). After reduction neither HA receptor is able to bind HA (lanes 3 & 4).

Figure 3. Effects of reduction and endoglycosidase-F treatment on composition of the purified 175 kDa HAR and 300 kDa HA receptors. HA receptors were immuno-affinity purified as in Fig. 1 and analyzed by SDS-PAGE before and after treatment with N-glycosidase F and/or reduction with β -mercaptoethanol as indicated. Open and solid arrows indicate, respectively, the positions of the untreated 175 kDa and 300 kDa HA receptors. The four nondeglycosylated proteins (lane 3, labeled a-d with arrowheads) generated by reduction of the purified HA receptors are all shifted to lower apparent size by enzyme treatment.

Figure 4. The 175 kDa HA receptor contains a single protein, but the 300 kDa HA receptor contains three proteins. LEC HA receptors were immunopurified as in Fig. 1, subjected to SDS-PAGE and the 175 kDa HA receptor (lanes 3 & 4) and 300 kDa HA receptor (lanes 1 & 2) bands were excised, and rerun on a 7% gel with or without reduction as indicated. The position of the nonreduced 300 kDa and 175 kDa HARs are indicated by the solid or open arrows, respectively. Three proteins of approximately 97 kDa, 230 kDa and 260 kDa (solid arrowheads a, c & d; compare to Fig. 3, lane 3) arise from reduction of the 300 kDa HA receptor (lane 2). From high to low mass, respectively, these three subunits are designated α , β , and γ . The 175 kDa HA receptor gives a single ~185 kDa species (open arrowhead b) after reduction (lane 3). This 185 kDa protein is not seen in the 300 kDa HA receptor.

Figure 5. Model for the structure of LEC HA receptors. The scheme summarizes the organization and composition of the affinity purified HA receptor from rat LECs. The numbers indicate the approximate mass in kDa of each protein. HA receptor preparations may contain two independent HA isoreceptors or may be a super large complex composed of two (or three) copies of the 175 kDa protein and one copy of the 300 kDa HA receptor complex. The 300 kDa HA receptor is a heterotrimeric complex of three subunits (α , β and γ) that are disulfide-bonded. Although the model shows each of the three subunits disulfide-linked to the other two, this is not yet known.

Table I

Affinity purified LEC HA receptor preparations were first reduced with 5% (v/v) β -mercaptoethanol and then analyzed by SDS-PAGE followed by staining with silver as described in Experimental Procedures. Stained protein bands were quantitated using a Molecular Dynamics Personal Densitometer. The total staining intensity for the reduced 175 kDa protein (which migrates at 185 kDa) and the three bands derived from the 300 kDa HA receptor was set at 1.0. The fraction of total staining in each of the four bands is shown.

Experiment	Protein Band			
	185 kDa	97 kDa	230 kDa	260 kDa
	(fraction of the total staining)			
1	0.43	0.14	0.24	0.19
2	0.46	0.05	0.21	0.28
3	0.51	0.10	0.24	0.15
Mean:	0.47 ± 0.04	0.10 ± 0.05	0.23 ± 0.02	0.21 ± 0.07
Mole ratio:	2.5	1.0	1.0	0.8

T

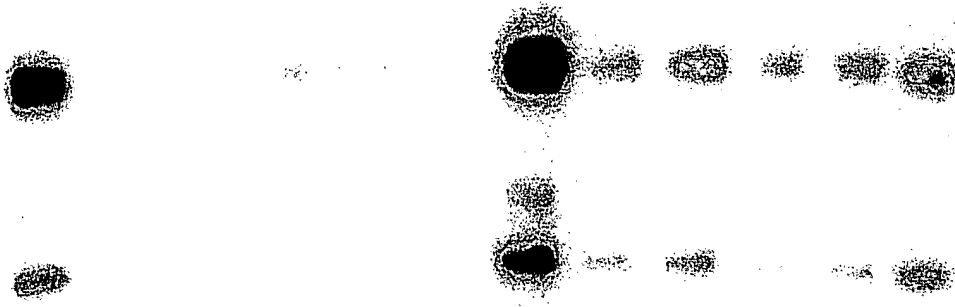


Fig 1 in
original submission
to PLoS ONE
on 4-25-99

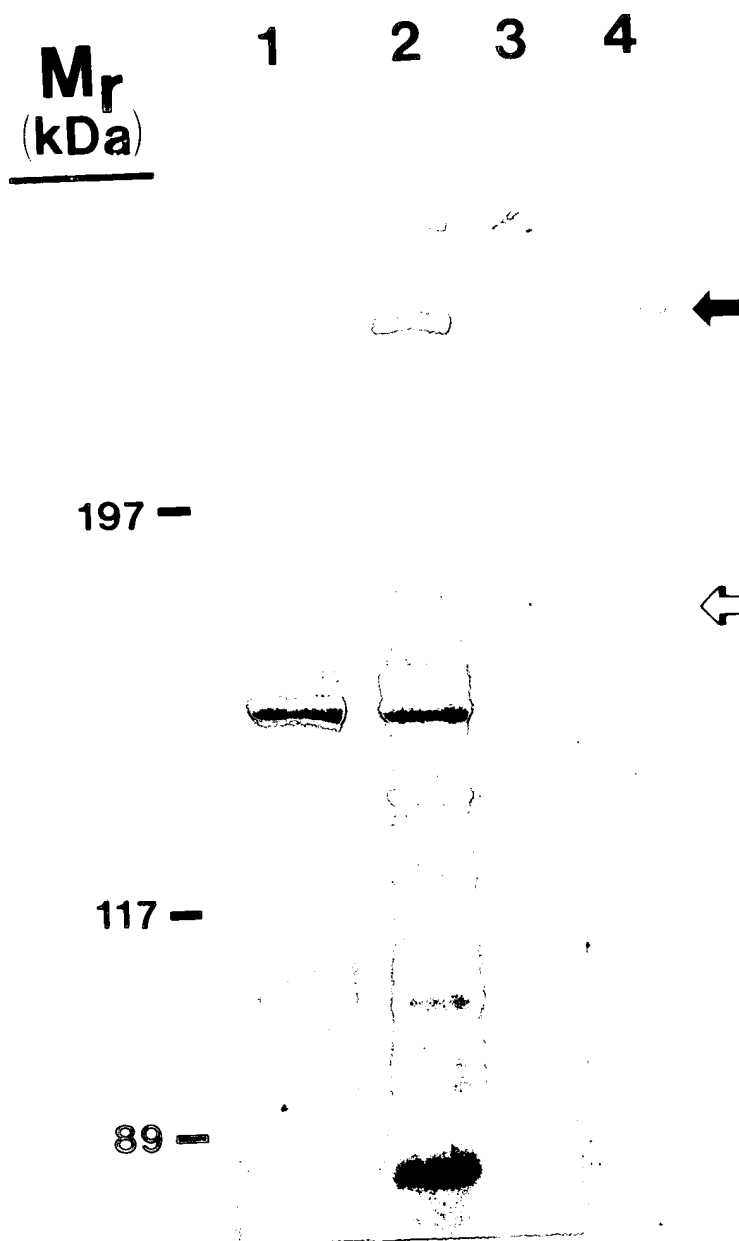
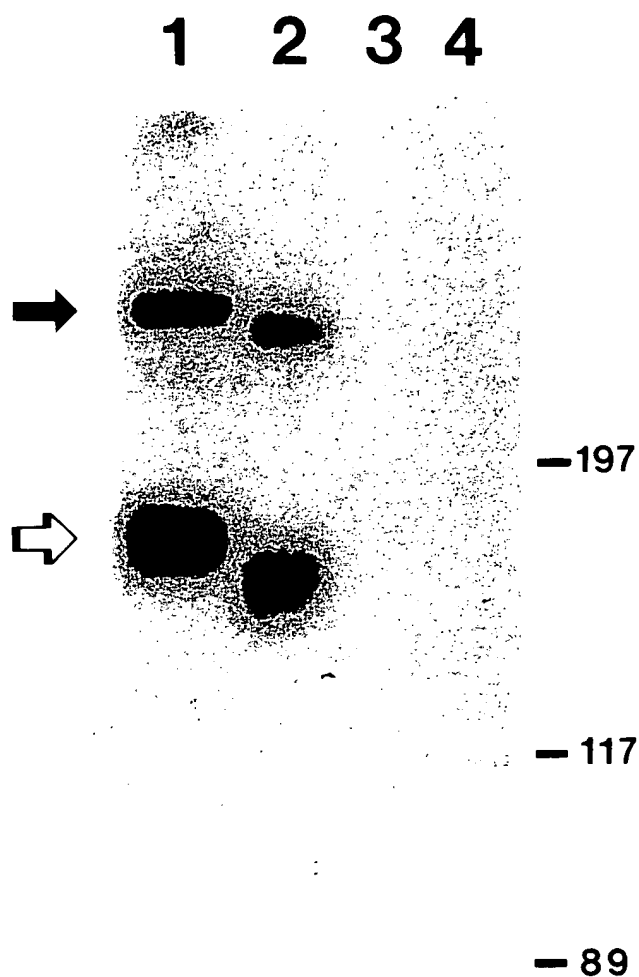


Fig 2 in Brocke MS

Fig 2 in final IBC
paper

Fig 1 in original IBC



PNGaseF:	-	+	-	+
β -ME:	-	-	+	+

Fig 5 in Blackwell
 Fig 3 in final JBC
 paper
 Fig 2 in original JBC

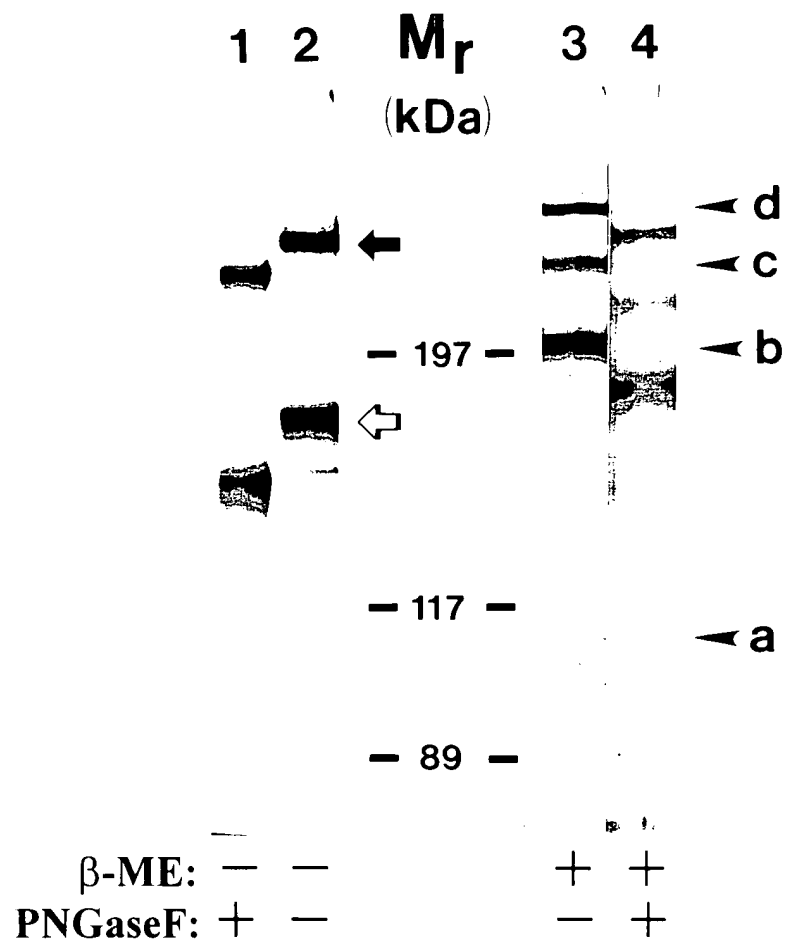


Fig 3 in Blocker MS

Fig 4 in final JBC
paper

Fig 3 in orig JBC

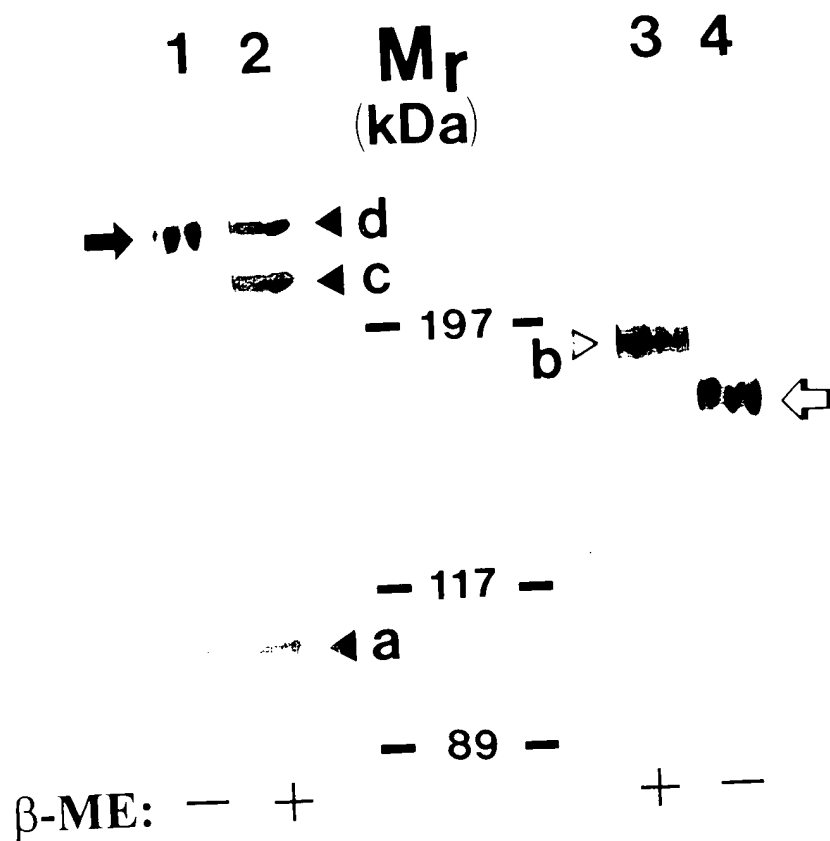


Fig 4 in Biochem MS

Fig. 5 in final JBC
paper

Fig 4 in orig JBC

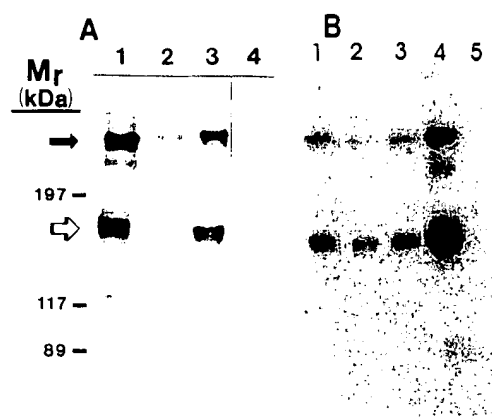


Fig 1 in
final JBC
paper

Zhou, et al. Fig 1
art. # 3345

$$\left. \begin{array}{l} \text{ } \\ \text{ } \\ \text{ } \end{array} \right\}$$


Super-large HAR Complex

paper

495 in original JBC

ATTACHMENT "B"

July 23, 1999

Herbert Tabor
Editor, JOURNAL OF BIOLOGICAL CHEMISTRY
9650 ROCKVILLE PIKE
BETHESDA, MD 20814

Dear Dr. Tabor:

Enclosed is a manuscript entitled "Purification and subunit characterization of the rat liver endocytic hyaluronan receptor" by Bin Zhou, Janet A. Oka, Anil Singh and Paul H Weigel, which we are submitting for consideration as a **Communication**. The original glossy figures and four copies of the manuscript are included as well as a disk containing PC Word97 files for the manuscript and cover letter. Members of the Editorial Board with appropriate expertise as possible reviewers are D. Carlson, H. E. Conrad, N. Dahms, G. Hart, P. Stahl and S. Kornfeld. Investigators with a conflict of interest, who should not be considered as reviewers include Drs. Bard Smedsrod, Torvard Laurent, Peter McCourt and Staffan Johansson.

The hyaluronan receptor was discovered in mammals in the early 1980s but has not yet been successfully purified or cloned from any source. This is the first report of a purification of this endocytic clearance receptor, which removes circulating hyaluronan and chondroitin sulfate fragments from the blood. In this study we determine the subunit composition of the purified rat liver HA receptor, that all subunits contain N-linked oligosaccharides, that the deglycosylated receptor still binds HA and that the reduced proteins are unable to bind hyaluronan.

The reviewers may require additional information, since the present manuscript utilizes a monoclonal antibody to be described in another manuscript that is still in preparation. We have therefore, included **Supplemental Information for Reviewers** as an addendum to the manuscript. A copy is attached to this letter as page 2. If necessary, we will provide these additional data to the reviewers. However, citing these additional results in the present paper would likely be inappropriate because there are length restrictions and there would be too many "data not shown" statements.

Despite the apparent complexity of the HA receptor system, we feel it is appropriate, timely and very important to the field to publish the first purification of the *bone fide* receptor as quickly as possible. The present report will also correct a previous erroneous claim by others that this hyaluronan receptor is identical to ICAM-1.

We look forward to your response.

Sincerely,

Paul H. Weigel, Ph.D.

Supplemental Information for Reviewers of

“Purification and subunit characterization of the rat liver endocytic hyaluronan receptor”

We recognize that the reviewers may require additional information, since the present manuscript utilizes a monoclonal antibody to be described in another manuscript that is still in preparation. The following summary of results from ongoing studies may answer some questions or clarify points that need to be addressed for review of the manuscript. If the reviewers feel they need to see some of these additional data in order to assess the paper, we will gladly provide it.

* * * * *

We have generated eight monoclonal antibodies against the rat LEC 175 kDa HA receptor, which had been partially purified by RCA-I chromatography and preparative SDS-PAGE. Two of the antibodies block HA binding to live cells and to both purified HA receptors in the ligand blot assay, verifying that the authentic HA receptor is being recognized. All antibodies against the 175 kDa HA receptor cross-react with the ~300 kDa HA receptor in Western and/or immunoprecipitation studies. These anti-175 kDa monoclonal antibodies cross-react with the large α and β subunits of the 300 kDa receptor. This surprising degree of cross-reactivity at first seems inconsistent with our present conclusion that the 300 kDa species is not a dimer of the 175 kDa species and does not contain the 175 kDa species as a subunit. These results, however, are explained by the following data.

Based on six peptide sequences from the affinity purified 175 kDa species, we have isolated a novel partial cDNA of >5 kb that verifies all six peptide sequences. Surprisingly, even though the 5' end of this cDNA is still incomplete, it encodes a core protein of almost 200,000, which is much larger than the 175 kDa receptor. Furthermore, using three different portions of this cDNA sequence (representing both ends and the middle) as a probe in Northern analysis of LEC mRNA, we detect only a single species. The size of this mRNA is far greater at ~10 kb than the expected size for the 175 kDa species (<6 kb). Therefore, there appears to be no mRNA for a 175 kDa HA receptor species.

These results initially appeared inconsistent with the results in the present study until we sequenced the N-terminus of the affinity purified 175 kDa HA receptor and found staggered termini, whose major sequence matches the partial cDNA and give the predicted 175 kDa size. Therefore, we presently believe that the 175 kDa HA receptor is actually derived from a much larger protein, either the largest α -subunit of the ~300 kDa receptor or a larger precursor from which most of the HAR subunits may be derived. Further data, such as comparative peptide maps of the various subunits and completion of the cDNA cloning, are needed to confirm this conclusion. These studies are in progress.

* * * * *

Citing these additional results in the present paper would likely be difficult due to length restrictions and because there would be many “data not shown” statements. However, if the reviewers believe it is important or appropriate to include some of these points in the text, we will do so.

ATTACHMENT "C"

Paul H. Weigel

From: Judy Christopher [jchristo@asbmb.faseb.org]
 Sent: Tuesday, August 24, 1999 11:40 AM
 To: paul-weigel@OUHSC.edu
 Subject: From the JBC re: Communication C9:00621

C9:00621
 Dear Dr. Weigel:

Your communication, "Purification and subunit characterization of the rat liver endocytic hyaluronan receptor," has been reviewed by a member of the Editorial Board whose critique is attached. I am pleased to say the reviewer considered the paper important and novel, and suitable for publication in The Journal of Biological Chemistry after revision. The reviewer felt that it is critical to include experiments defining the specificity of the antibody used, even if this addition exceeds the page limit for a Communication. If so, the reviewer recommends publication as a regular paper with the additional information. The reviewer also asks that a minor clarification concerning a previous publication be included.

M.M. 30 Western

I anticipate that you will be able to address these concerns and submit a revised version of your manuscript. The JBC is now accepting electronic submissions for all manuscripts. When you return your manuscript, please go to the JBC Online Submission Site to submit your manuscript. The site is quite straightforward, and you will be asked to submit your contact information, a cover letter, text files and graphic files. Please see the Instructions to Authors for preparation of text and graphic files (<http://www.jbc.org/misc/itoa.shtml>). Please be sure to indicate that your manuscript is a revision of a former JBC manuscript and provide the old manuscript number. The address for the site is:

<http://171.64.249.91/asbmbosrs.nsf/home?readform>

If for any reason you are not able to submit your manuscript electronically, please forward your revisions directly to the Bethesda office. Include a cover letter with detailed responses to the reviewers' comments, three copies of the revised manuscript with at least one set of original figures. A complete electronic copy of the manuscript on disk, with files for both text and graphics must also be included. Send these materials to:

The Journal of Biological Chemistry
 9650 Rockville Pike
 Bethesda, MD 20814

Any questions should be directed to: jbc@asbmb.faseb.org

Thank you for allowing the Journal to review your work. Thank you for allowing the Journal to review your work.

Sincerely,

Vincent C. Hascall
 Associate Editor

COMMENTS FOR AUTHORS:

This is a very interesting description of the identification of an endocytosis receptor for hyaluronic acid. The paper clears up considerable confusion about the identity of the receptor and will be of wide interest to the readership of the Journal. Unfortunately it is not possible to judge the work without more information on the antibody. I would encourage the authors to include the antibody work in the paper even if it means scaling up to a full paper. It will also be important

to demonstrate that the receptor in question is actually an endocytosis receptor. The antibodies would be suitable to make this point. >

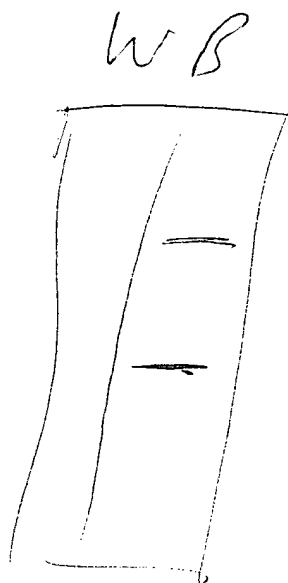
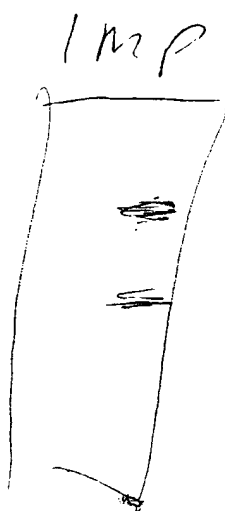
A second point that needs to be addressed is the statement on Page 9, 3rd paragraph, last line where it is ~~stated that~~ the earlier claim that ICAM is the HA receptor ~~has been withdrawn~~. A cursory look at the McCourt and Gustafson paper (Int. J. Biochem. Cell Biol. 29, 1179 (1997)) failed to identify language that a retraction was made. It would probably be prudent for the authors to rewrite this sentence.

deleted statement

Imp of active HARE to MoAb 3C ✓

WB ✓

+ control



Paul H. Weigel

From: Judy Christopher [jchristo@asbmb.faseb.org]
Sent: Wednesday, August 25, 1999 8:19 AM
To: paul-weigel@OUHSC.edu
Subject: From the JBC re: Communication C9:00621

C9:00621

Dear Dr. Weigel:

In answer to the quesitons in your e-mail:

1. If you are returning a hard copy of the manuscript rather than submitting electronically, it goes to Bethesda in order to be put into OSRS. The Editorial Board member handling the review of your Communication indicated the desire to see the paper after revision, so it will have to go into OSRS, either by you submitting it online or the hard copy and the disks being sent to Bethesda.
2. Your original manuscript was put into the OSRS and the figures scanned in. I am not sure of the protocol on resubmitting manuscripts. You should go to the site:

<http://171.64.249.91/asbmbosrs.nsf//home?readform>

which will give you instructions on how to proceed. Please note that your questions about OSRS should be sent to:

jbc@asbmb.faseb.org

Unfortunately, neither Dr. Hascall nor I are experts on the use of the OSRS.

Sincerely,

Judy Christopher
Secretary to Vincent C. Hascall
Associate Editor

August 30, 1999

Vincent C. Hascall, Ph.D.
Associate Editor
JOURNAL OF BIOLOGICAL CHEMISTRY
c/o 9650 ROCKVILLE PIKE
BETHESDA, MD 20814

Dear Dr. Hascall:

Thank you for your letter of August 24th, indicating the favorable review of our manuscript (MS# C9-00621) entitled "Purification and subunit characterization of the rat liver endocytic hyaluronan receptor" by Bin Zhou, Janet A. Oka, Anil Singh and Paul H Weigel. We thank the reviewer for his/her thoughtful comments. Since the revised manuscript is being submitted via email, no hard copies are included. The submission includes PCWord97 files for this cover letter and the revised manuscript as well as Corel Paint TIFF files for a new Figure 1 as well as the original Figures 1-5, which are renumbered, respectively, as Figures 2-6.

The Communication has been revised as suggested in your letter and the reviewer's comments.

1) In response to the reviewer's request for more information on the monoclonal antibody used in this study, we have included an additional figure (new Figure 1), discussed on page 7. Since an additional figure was added, the remaining original figures 1-5 were renumbered as Figures 2-6. This new figure shows Western blot and ligand blot data to demonstrate that the antibody (designated 175HAR-30) recognizes the *bone fide* HA receptor. Panel A shows that this antibody identifies the 175kD and 300kD receptor species in Western blots and quantitatively removes both proteins from extracts during immunopurification. Panel B shows in a separate experiment that the HA binding activity of the two receptor species, measured in the ligand blot assay, follows the two proteins; removal of the proteins also removes the HA binding activity. We believe that this additional information satisfactorily documents the validity of using this monoclonal antibody.

2) In response to the request for information that the protein identified is the endocytic HA receptor, we have included a statement (page 7) about the effect of a second monoclonal antibody, designated 175HAR-174, that completely inhibits endocytosis in live cells. Like 175HAR-30, this second antibody also recognizes the two HA receptor species in Western blots

and immunoprecipitates both proteins and all the HA binding activity. Unlike 175HAR-30, the 174 antibody also inhibits endocytosis of 125 I-HA mediated by the receptor.

3) The sentence noted on page 9 of the original manuscript was altered as suggested by the reviewer by deleting the phrase about withdrawal of the report.

We hope that the reviewer finds these changes acceptable. Although it is not feasible to include all the relevant and supporting data regarding the preparation and characterization of monoclonal antibodies in this report, we believe the additional figure strengthens the paper and confirms that the antibody is against the HA receptor.

We have also shortened the paper throughout, in order to accommodate the additional figure and still be within the page restriction for a Communication. The original version had 4169 words with 25,787 characters and spaces; the revised version has <4000 words and <25,100 characters and spaces. It is very important to us that these results be published in the Journal as quickly as possible as a Communication.

Thank you for your assistance. We look forward to your response.

Sincerely,

Paul H. Weigel, Ph.D.
TEL: (405) 271-1288
FAX: (405) 271-3092
email: paul-weigel@OUHSC.edu



ATTACHMENT "D"

PUBLISHED BY THE
AMERICAN CHEMICAL SOCIETY

Duke University Medical Center
081 Yellow Zone, Duke South
DUMC 3673
Durham, North Carolina 27710
USA
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International Fax: (919) 681-8344
Domestic Fax: 1-800-334-0892

Gordon G. Hammes. *Editor*

July 7, 1999

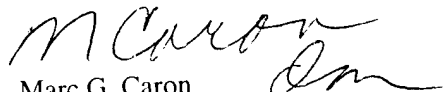
Dr. Paul H. Weigel
Dept. Biochemistry & Molecular Biology
Univ. of Oklahoma Health Sciences Center
PO Box 26901
Biomedical Sciences Bldg, Rm 853
Oklahoma City OK 73190

Re: BI991023X
**Purification and Characterization of the Endocytic Hyaluronan Receptor From
Rat Liver Sinusoidal Endothelial Cells**

Dear Dr. Weigel:

Your manuscript, referenced above, has been examined by two expert referees. The two reviewers reached different conclusions. In such cases, the manuscript is sent to a member of the Editorial Advisory Board for arbitration. Unfortunately, the EAB member sided with the more critical reviewer and recommended rejection. For reasons detailed in their enclosed reviews, they feel that the paper is inappropriate for publication in BIOCHEMISTRY. We nevertheless appreciate your decision to submit the work and would be pleased to consider other manuscripts from your laboratory.

Sincerely yours,


Marc G. Caron
Associate Editor

Enclosures:
Copyright Transfer Form (if received)
Reviewer Comments



The University of Oklahoma
Health Sciences Center

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

Paul H. Weigel, Ph.D.
Professor and Chairman

April 28, 1999

Gordon G. Hammes, Ph.D.
Editor, BIOCHEMISTRY
Duke University Medical Center
Box 3673, 081 Yellow Zone, Duke South
Durham, NC 27710

Dear Dr. Hammes:

Enclosed is a manuscript entitled "Purification and characterization of the endocytic hyaluronan receptor from rat liver sinusoidal endothelial cells" by Bin Zhou, Janet A. Oka, Anil Singh and Paul H Weigel, which we would like to be considered as a regular paper. The original, three copies and a copyright form are included. The following are possible reviewers with expertise investigating membrane receptors or binding proteins that recognize carbohydrate ligands, including hyaluronan: Jacques Baenziger (Washington University), Phil Stahl (Washington University), Warren Knudsen (Rush-Presbyterian-St Luke's Medical Center, Chicago), Bryan Toole (Tufts), and Douglas McAbee (Cal State-long Beach).

The hyaluronan receptor was discovered in mammals in the mid-1980s but has not yet been successfully purified or cloned from any source. This is the first report of a purification of this endocytic clearance receptor, which removes circulating hyaluronan and chondroitin sulfate fragments from the blood. We have characterized the subunit composition of the purified rat liver HA receptor, determined that all subunits contain N-linked oligosaccharides, that the deglycosylated receptor still binds HA and that the reduced proteins are unable to bind hyaluronan. The present report also corrects a previous erroneous claim by others that this hyaluronan receptor is identical to ICAM-1.

We look forward to your response.

Sincerely,

Paul H. Weigel, Ph.D.

TEL: (405) 271-1288

FAX: (405) 271-3092



The University of Oklahoma
Health Sciences Center

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

Paul H. Weigel, Ph.D.
Professor and Chairman

VIA FACSIMILE: (800) 334-0892

June 25, 1999

Attention: Doris Hunte

Marc G. Caron, Ph.D.
Associate Editor, BIOCHEMISTRY
Duke University Medical Center
Box 3673, 081 Yellow Zone, Duke South
Durham, NC 27710

Dear Dr. Caron:

In my previous submission letter of April 28th for manuscript BI99 1023 (entitled: "Purification and characterization of the endocytic hyaluronan receptor from rat liver sinusoidal endothelial cells" by Bin Zhou, Janet A. Oka, Anil Singh and Paul H Weigel), I neglected to indicate several investigators with conflicts of interest, who should not be considered as reviewers. These include Drs. Bard Smedsrod, Torvard Laurent, Peter McCourt and Staffan Johansson. Although you receive this request too late to accommodate changes in the selection of reviewers, I thought I should mention these exclusions, especially after learning that the manuscript has been sent out for additional review.

Sincerely,

Paul H. Weigel, Ph.D.
TEL: (405) 271-1288
FAX: (405) 271-3092

BIOCHEMISTRY

←Reviewer No.

Manuscript No. →
Corresponding →
Author
Abbreviated →
Title

BI99 1023 X-9-a-0

67

Bin Zhou, Janet A. Oka, Anil Singh, Paul H. Weigel

Purification and Characterization of the Endocytic
Hyaluronan Receptor From Rat Liver Sinusoidal
Endothelial Cells

Ms. Type: a:article

Comments:

This manuscript reports on the purification of the endocytic receptor for hyaluronan from rat liver sinusoidal endothelial cells. A model of the receptor as a protein complex is proposed. The findings are interesting and may finally define the receptor. The key to the purification and the identification was the use of monoclonal antibodies prepared against the 175 kDa band. Unfortunately, the characterization and the action of the antibodies were left to two other manuscripts in preparation and to be submitted elsewhere. It is particularly puzzling why a monoclonal antibody would recognize both a 175-kDa and a 300-kDa protein when the latter was said to be not a dimer of the 175-kDa protein. A further puzzle is why both species would bind the radioactive HA. This reviewer felt therefore the present manuscript does not contain adequate experimental information for evaluation and at best is incomplete as a full paper.

AUTHOR'S ANONYMOUS COPY

2 of 2 66

Ms# BI991023X

Title: Purification and characterization of the endocytic hyaluronan receptor from rat liver sinusoidal endothelial cells
Authors: Zhou, Oka, Singh and Weigel

This manuscript describes purification of the liver endocytic receptor for hyaluronan and presents a model for its subunit composition. This manuscript represents the culmination of much previous work by this group and provides a definitive identification of the protein species that form the receptor(s). Sequencing/cloning, however, has not yet been achieved.

The experiments presented are well performed and in my opinion this work should definitely be published. However, the style in which this manuscript is written is not optimal. The main problem is with the Results section which is presented in a chatty manner that must be confusing for anyone not familiar with the history of this project. I am quite familiar with the history but still found it confusing, sometimes annoying. I believe the authors took this approach to help people to understand the background to their study but I disagree with their tactics.

The opening section of the Results serves no obvious purpose and should be eliminated. Simply describe the method used for purification as appears in the next two sections. Keep it simple and present the purification without embellishments like "the beauty of a ligand blot---" and discussions of past mistakes (ICAM etc).

Discussion of ICAM etc belongs in the Discussion section. However the Discussion section is far too long. This manuscript presents important but very straightforward data. Discuss it that way. Shorten the whole manuscript as though it were a brief communication.

BIOCHEMISTRY

←Reviewer No.

Manuscript No. →
Corresponding →
Author
Abbreviated →
Title

BI99 1023 X-9-a-0

65

Bin Zhou, Janet A. Oka, Anil Singh, Paul H. Weigel

Purification and Characterization of the Endocytic
Hyaluronan Receptor From Rat Liver Sinusoidal
Endothelial Cells

Ms. Type: a:article

Comments:

This paper describes the purification of one or more hyaluronan receptors from liver sinusoidal endothelial cells. It is believed that this receptor accounts for the hepatic clearance of hyaluronic acid and other glycosaminoglycans from the circulation. Purification is based on isolation of endothelial cells, enrichment over a lectin column, and final purification by affinity chromatography using a monoclonal antibody originally raised to the 175 kDa form of the receptor. Although this reviewer understands that this has been an elusive and difficult to purify receptor, the present manuscript is not particularly satisfying due to the highly preliminary nature of the studies reported and the absence of much of the supporting evidence that this is indeed the endocytic hyaluronan receptor. No evidence is given in this manuscript that the monoclonal antibody utilized is directed at the hyaluronan receptor. It is noted in the discussion that the antibody does block binding and uptake by isolated cells, however, this information as well as the information about the monoclonal antibody itself are to be published elsewhere. Thus, one must take on faith the results reporting the relationship to this receptor and hyaluronan uptake. In addition, no binding studies are provided, nor any indication as to yield or fold. No amino acid sequence or peptide mapping data is provided to give an independent indication of the relationship between the various bands obtained. Further, the molecular weights proposed for the 300 kDa form, which presumably consists of disulfide linked subunits and the molecular weights of the subunits is highly unsatisfying with no compelling evidence that the proposed relationships are correct. As a result, the studies, if correct, are potentially very interesting and suggest a protein with very unusual properties and the possibility of two rather than one distinct form of hyaluronan receptor. However, the studies are of such a preliminary nature and so incomplete that this reviewer does not feel they would be suitable for publication in *Biochemistry*, in part because they do not represent a significant advance without the supporting data and characterization. This reviewer must reluctantly recommend declination of this manuscript despite the authors having made some advances on what has admittedly been a very difficult problem. If the authors are willing to provide a more complete study with better documentation and characterization, it would certainly be appropriate for publication in *Biochemistry*.

AUTHOR'S ANONYMOUS COPY



April 28, 1999

PURIFICATION AND CHARACTERIZATION OF THE ENDOCYTIC HYALURONAN

RECEPTOR FROM RAT LIVER SINUSOIDAL ENDOTHELIAL CELLS

Bin Zhou, Janet A. Oka, Anil Singh and Paul H. Weigel *

Department of Biochemistry & Molecular Biology

University of Oklahoma Health Sciences Center

Oklahoma City, OK 73190

* To whom correspondence should be addressed,

TEL: (405) 271-1288

FAX: (405) 271-3092

email: paul-weigel@OUHSC.edu

Running Title: Purification of the LEC endocytic HA receptor

Key words: hyaluronic acid, hyaluronan binding protein, receptor mediated endocytosis, recycling receptor, iso receptors

ABSTRACT

The endocytic hyaluronan (HA) receptor of liver sinusoidal endothelial cells (LECs) is responsible for the clearance of HA and other glycosaminoglycans from the circulation in mammals. This membrane-bound HA receptor has not yet been purified or cloned. We report here for the first time the purification of the *bone fide* rat liver HA receptor. Using lectin and immuno-affinity chromatography, two HA receptor species were purified from detergent-solubilized membranes prepared from purified LECs. In nonreducing SDS-PAGE, these two proteins migrated at 175 kDa and ~ 300 kDa corresponding to the two species previously identified by photoaffinity labeling of live cells as the HA receptor (Yannariello-Brown, et al. *J. Biol. Chem.* **267**, 20451, 1992). These two proteins co-purify in a molar ratio of 2:1 (175:300) and both proteins are active, able to bind HA after SDS-PAGE, electrotransfer and renaturation. After reduction, the 175 kDa protein migrates as a ~185 kDa protein and is not able to bind HA. The 300 kDa HA receptor is a complex of three disulfide-bonded subunits that migrate in reducing SDS-PAGE at ~ 260, 230 and 97 kDa. These proteins designated, respectively, the α , β , and γ subunits are present in a molar ratio of 1:1:1 and are also unable to bind HA when reduced. Both the 175 kDa and 300 kDa species contain N-linked oligosaccharides, as indicated by a 25-30 kDa decrease after treatment with endoglycosidase-F, but the deglycosylated nonreduced proteins still bind HA.

Hyaluronan (HA¹) is an important component of the extracellular matrix of all tissues and plays an important role in many developmental and regulatory processes such as cell proliferation, cell adhesion, recognition, morphogenesis, differentiation and inflammation (1-4). The daily total body turnover of HA in humans is estimated to be at least 1 g/day (5). HA degradation and removal in the body occurs via two clearance systems (3): one is in the lymphatic system, which accounts for ~ 85% of the HA turnover, and another system is hepatic, accounting for ~15% of the total body HA turnover. HA in the extracellular matrices of tissues throughout the body is continuously being synthesized and degraded. Very large HA molecules (~10⁷ Da) are partially degraded to large fragments (~10⁶ Da) that are then released from the matrix and flow with the lymph to lymph nodes. The majority of HA (~85%) is completely degraded in the lymph nodes by unknown cell types and unknown mechanisms and the remaining HA (~ 15%) that passes through the nodes finally enters the blood. Clearance of this circulating HA is presumably very important for normal health (3-5). Elevated serum HA levels are found in several disease conditions such as liver cirrhosis, rheumatoid arthritis, psoriasis, scleroderma and some cancers (6-8).

The liver sinusoidal endothelial cells (LECs) have a very active recycling, endocytic receptor that removes HA and other glycosaminoglycans, such as chondroitin sulfate, from the circulation (3, 4, 9-11). An earlier report misidentified this LEC HA receptor as ICAM-1 (12,13), also known as CD54, which is a 90 kDa protein (14). This finding was later recognized as an artifact in that ICAM-1 bound nonspecifically to the HA affinity resin employed in the putative purification (15).

In two previous studies, one using a photoaffinity derivative of HA (16) and the other using a novel ligand blot assay with ¹²⁵I-HA (17,18), we identified two specific HA-binding proteins in isolated rat LECs at 175 kDa and 300 kDa. In the present study, we have finally

purified these two proteins for the first time. Our results show the 300 kDa HA receptor protein contains three subunits after reduction but does not contain the 175 kDa HA receptor protein, which itself contains no other subunits.

MATERIALS AND METHODS

Materials—Lectin gel kit and RCA-I-agarose gel were purchased from EY laboratories, Inc. Copper phthalocyanine tetrasulfonic acid tetrasodium salt (CPTS) was obtained from Aldrich Chemical Co. Trishydroxymethylamino methane, SDS, ammonium persulfate, N, N'-methylenebisacrylamide, and SDS-PAGE molecular weight standards were from Bio-Rad. Na¹²⁵I was from Amersham Corp. NP-40 was from CalBiochem and 1,3,4,6-Tetrachloro-3 α ,6 α -diphenylglycouril (iodogen) was from Pierce. HA (human umbilical cord) obtained from Sigma, was further purified as described previously by fractionation on celite and ethanol precipitation (19). Nitrocellulose membranes were from Schleicher & Schuell. Acrylamide and urea were from U.S. Biochemical Corp. p-Nitrophenylphosphate was from Kirkegaard & Perry Laboratories. All other chemicals were reagent grade and were from Sigma. Tris-buffered saline (TBS) contains 20 mM Tris-HCl, pH 7.0, 150 mM NaCl.

Preparation of LECs and LEC membranes—Male Sprague Dawley rats were purchased from Harlan, Indianapolis, IN. Liver endothelial cells were isolated by a modified (20) collagenase perfusion procedure (21), followed by differential centrifugation and then discontinuous Percoll gradient fractionation. Cells were collected from the 25/50% interface and washed 3-times with PBS at 4°C. For preparation of LEC membranes, the cells were hypotonically swollen, homogenized, and centrifuged at 1000xg to remove debris. The supernatant material was then centrifuged at 105,000 xg to obtain the total membrane fraction (22).

Ligand blot assay. LECs, membranes or protein samples were solubilized in an SDS sample buffer containing 16 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% glycerol (v/v), and 0.01% bromophenol blue (23). No reducing agent was added unless as noted in the figures. Cell or membrane samples were sonicated on ice for 10-20 s. After SDS-PAGE, the contents of the gel were electrotransferred onto a 0.1 μ m nitrocellulose membrane for 2 h at 24 V at 4°C using 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol and 0.01% SDS. The nitrocellulose membrane was treated with TBS containing 0.05% Tween-20 (TBST) at 4°C for 2 h or overnight and then incubated with 2 μ g/ml 125 I-HA in TBS without or with a 150-fold excess of HA (as competitor) to assess total and nonspecific binding, respectively. The nitrocellulose membrane was washed 5-times for 5 min each with 0.05% Tween-20 in TBS and dried at room temperature. The 125 I-HA bound to protein was detected by autoradiography with Kodak BioMax film. Nonspecific binding in this assay is typically <5%

Purification of the HA receptor. Total LEC membranes from eighteen rats were suspended in 200 μ l TBS containing 2% NP-40 (v/v) and gently mixed by rotation at 4°C for 2 h. The solubilized membranes were then diluted with TBS to a final NP-40 concentration of 0.5% and centrifuged for 30 min at 100,000xg. The supernatant extract was then loaded at room temperature onto a RCA-I gel column containing 10 ml of resin. The column was washed with 10 volumes of TBS containing 0.05% Tween 20. The bound proteins were eluted with 100 mM lactose in distilled water. The eluate was dialysed (using dialysis tubing) against multiple changes of TBS at 4°C overnight, concentrated about 10-fold using a Centricon-30 device (from Amicon), and then passed slowly over an immuno-affinity column (~8 ml), containing monoclonal antibody 175HAR-30 coupled to CNBr-activated Sepharose (~ 2 mg/ml resin). Details on the preparation and characterization of this and other monoclonal antibodies raised against the rat LEC 175 kDa HAR will be described elsewhere². The affinity column was washed with 10 volumes of 0.05%

Tween-20 in TBS and then eluted with 100 mM sodium citrate, pH 3.0. Eluted fractions were immediately neutralized by collection into 1 M Tris. The eluted fractions containing protein were pooled, dialysed against TBS at 4°C overnight and then concentrated using a Centricon-30. Samples of the concentrated solution were subjected to SDS-PAGE and HAR activity was detected by the ^{125}I -HA ligand-blot assay.

HA receptor binding to different lectins. LEC membrane extract (50 μl) was added to 100 μl of each of 9 different lectin-agarose gels in 0.5 ml microcentrifuge tubes. The tubes were incubated at 4°C with gentle rotation for 2 h and the gels were washed 4 times by centrifugation with 500 μl of 0.05% Tween-20 in TBS. The bound proteins were then either eluted with different mono- or disaccharides according to the specificity of the lectin or directly eluted with SDS sample buffer. The eluted solutions were subjected to SDS-PAGE and HA receptors were detected by the ^{125}I -HA ligand blot assay as described above. Proteins were visualized by staining with silver (24) or Coomassie blue.

Deglycosylation of HA receptors with glycopeptidase F. Purified HA receptor (1.17 μg) was treated with 0.5% SDS at 4 °C for 2 h and then at 90 °C for 3 min. Samples (22 μl) were chilled on ice for 4 min, and then 0.5 M Tris-HCl, pH 7.2 was added to a final concentration of 10 mM. Glycopeptidase F (25; 0.5 unit) and distilled water were added to give a final volume of 25 μl . The samples were incubated at 37°C overnight, 9 μl of 4-fold concentrated SDS sample buffer was added and they were heated for 3 min at 90°C. The samples were subjected to SDS-PAGE, and HA receptor protein was detected by silver staining or HA receptor activity was determined by the ^{125}I -HA ligand blot assay.

Two-dimensional electrophoresis. Affinity-purified HA receptor (~1.5 μg) was subjected to SDS-PAGE without reduction and the gel was stained with Coomassie blue. The 175 kDa and 300 kDa proteins were excised from the gel and the gel pieces were cut into smaller pieces. These gel

pieces were divided into two portions and incubated at 90°C for 4 min with SDS sample buffer with or without 10 mM DTT followed by 50 mM iodoacetamide. The samples were then subjected to a second dimension of SDS-PAGE without reducing agent in the gel. The proteins were visualized by silver staining (24) and HA receptor activity was assessed by the ¹²⁵I-HA ligand-blot assay.

General. Protein content was determined by the method of Bradford (26) using BSA as a standard. Receptor protein content was assessed after precipitation with 5% trichloroacetic acid to remove detergent. SDS-PAGE was performed according to the method of Laemmli (23). ¹²⁵I-HA was prepared using Iodogen (Pierce) and a uniquely modified hexylamine derivative of HA, which was synthesized as described previously (19) and purified by chromatography over a PD-10 column. HA was quantitated by measuring uronic acid content according to the procedure of Bitter and Muir (27). ¹²⁵I radioactivity was measured using a Packard Auto-Gamma Counting system.

RESULTS

A breakthrough in the ability to purify the LEC HAR occurred with our discovery that two very active and specific HA-binding proteins could be detected in LECs by ligand blotting using ¹²⁵I-HA (17,18). The 175 kDa and 300 kDa HA-binding species in LECs are readily detected after renaturation with Tween 20, even if the HAR activity in extracts had apparently been inactivated (as measured with a nondenaturing dot blot assay; 22). The LEC HAR is not very stable after solubilization, and the yield of HAR is low with most mild detergents (18,22), which is probably one reason for the earlier inaccurate report that ICAM-I is the LEC HAR (12,13). This report that the LEC HAR had been cloned has been partially retracted (15). The HA-binding activity at 175 kDa and 300 kDa in ligand blots corresponds perfectly to our previous identification of the HAR on intact LECs by the use of an HA photoaffinity derivative.

This HA probe specifically photo-labeled proteins of 175 kDa and 300 kDa (16). The two HAR species observed by ligand blotting with ^{125}I -HA also showed the same specificity with a panel of polyanionic competitors (18) as observed for intact LECs (10,11). The beauty of a ligand blot assay is that one can use any detergent to solubilize the membranes, since the HAR activity can be renatured after SDS-PAGE and electrotransfer.

Binding of the LEC HAR by lectins. We tested a battery of nine different lectin resins for their ability to bind and partially purify the LEC HAR activity (Fig. 1). Four lectins (HAA, PNA, LPA, and UEA-1) showed no ability to bind either HAR species. One lectin, SBA, gave variable results and was not pursued further. ConA, DBA WGA and RCA-I all appeared to remove active HARs quantitatively from LEC membrane extracts (Fig. 1A). As expected, a different profile of proteins was bound by each of these four lectins (Fig. 1B). RCA-I lectin was chosen for a purification scheme because HAR binding was quantitative, easily displaced with lactose and a significant purification from other membrane proteins was obtained. A further advantage of lectin chromatography as a purification step is the ability to elute the LEC HAR in a relatively small volume.

The final partial purification step for preparing the 175 kDa HAR as an antigen for monoclonal antibody production was preparative 1-D SDS-PAGE, which separated the 175 kDa HAR and 300 kDa HAR and gave fractions of discrete masses². Excellent correlation was observed between the position of stained protein bands and the HA-binding activity detected by the ligand blot assay. The highly purified and active 175 kDa fractions were pooled, concentrated, and used to prepare a panel of monoclonal antibodies, which will be described elsewhere².

Sequential lectin and immuno-affinity chromatography purifies the two LEC HAR species to homogeneity. Eight very useful monoclonal antibodies were obtained that recognize

the rat LEC 175 kDa HAR in Western blots after either nonreducing and/or reducing SDS-PAGE. The antibody 175HAR-30 was able to bind the 175 kDa HAR species, the original antigen, and also recognize the 300 kDa HAR species. However, as described below, the 300 kDa species is not a dimer of the 175 kDa protein (as we initially suspected; 16) and does not contain a 175 kDa subunit. This antibody was also able to immunoprecipitate both HAR proteins from LEC extracts. Therefore, the initial protocol to partially purify the 175 kDa HAR was modified by replacing the last 1-D electrophoresis step with affinity chromatography using this monoclonal antibody. This immuno-purification scheme enabled both the 175 kDa HAR and the 300 kDa HAR proteins to be purified for the first time (Fig. 2). Nonreducing SDS-PAGE analysis showed that these two proteins comprise >98% of the final purified HAR preparations. Based on silver and Coomassie blue staining of nonreduced gels, the 175 kDa HAR and 300 kDa HAR proteins are purified in an apparent molar ratio of 2:1 (Table I).

Subunit composition of the two HAR proteins. To determine if either protein contains disulfide-bonded subunits, the copurified 175 kDa HAR and 300 kDa HAR were analyzed by reducing SDS-PAGE (Fig. 3, lanes 3 and 4). After reduction with β -mercaptoethanol, four protein species were evident ranging in apparent size from 97 kDa to 260 kDa. In order to determine which HAR species yielded each of these four proteins, the 175 kDa HAR and 300 kDa HAR were first separated by nonreducing SDS-PAGE. The two HAR bands were then excised, and reanalyzed by SDS-PAGE with or without reduction with β -mercaptoethanol (Fig. 4). The reduced 175 kDa HAR yielded no other protein species, but the apparent size of the protein increased to ~185 kDa (Fig. 4, lane 3). This shift to higher M_r is typical of membrane receptors that contain disulfide bonds in their extracellular domains. The reduced 175 kDa HAR no longer binds ^{125}I -HA (Fig. 5, lane 3).

After reduction, the 300 kDa HAR gave rise to three protein species with apparent masses

of 260, 230 and 97 kDa, which we designate, respectively, as the α , β , and γ subunits of the 300 kDa HAR (Fig. 4, lane 2). None of these three reduced proteins were able to bind ^{125}I -HA (Fig. 5, lane 3). Based on Coomassie blue and silver staining, and their apparent sizes, the molar ratio of the three protein components of the 300 kDa HAR is 1:1:1 (Table II). In numerous purified HAR preparations, the stoichiometry of the 175 kDa and 300 kDa proteins has consistently been 2-3:1 (175:300). Therefore, the overall stoichiometry of the four proteins in reduced, affinity purified HA receptor preparations was 2:1:1:1, respectively, for the 175 kDa protein and the α , β , and γ subunits of the 300 kDa complex (Fig. 6).

Based on their sensitivity to endoglycosidase-F treatment, both HAR species contain multiple N-linked oligosaccharides (Fig. 3, lanes 1 and 2). During digestion with endoglycosidase-F, the 175 kDa HAR shows at least two intermediate bands (not shown), which were not well resolved, before being converted to a single ~150 kDa species, suggesting that at least 3 relatively large N-linked oligosaccharides are present. Surprisingly, the glycosidase-treated 175 kDa HAR, which was decreased in M_r by ~25 kDa, did not become more sharply focused; it is still apparently heterogeneous. The de-N-glycosylated 175 kDa HAR and 300 kDa HAR are still capable of specific ^{125}I -HA binding (Fig. 5). Therefore, N-linked oligosaccharides do not appear necessary for HA binding.

DISCUSSION

In addition to the normal turnover of HA in tissues throughout the body, a wide range of biomedical and clinical applications use exogenous HA that is also removed from the lymphatics or ultimately from the blood and degraded by the LEC HA receptor (3,5). For example, HA is used extensively in eye surgery (28), in the treatment of joint diseases including osteoarthritis (29) and is being developed as a drug delivery vehicle (30). Several years after we

proposed a role for HA in wound healing (31), numerous studies have explored the benefit of HA during this complex remodeling process (32,33). The exogenous HA introduced in these various applications is naturally degraded by the lymph and LEC systems noted above. Despite the very large endocytic and degradative capacity of the LEC HAR (34) and its importance in removing HA from the blood, the HA receptor had not yet been successfully purified.

In a previous study, we used a photoaffinity derivative of HA to identify two LEC surface proteins of 175 kDa and 300 kDa that were specifically labeled and, therefore, candidates for the HA receptor in these cells (16). At the time, we considered it likely that the 175 and 300 kDa species represented monomeric and dimeric forms, respectively, of the LEC HA receptor. Based on the present results, we now know this is not the case and that the 175 kDa HAR is not a covalently bound part of the 300 kDa HAR complex. Nonetheless, this photoaffinity approach identified the correct HAR proteins. Likewise, the ligand blot assay, which we developed subsequently to identify the LEC HAR (17,18), also monitors the same proteins that were purified in the present study. Our results, therefore, have consistently identified proteins of 175 kDa and ~ 300 kDa as the LEC HAR.

These points are relevant in light of earlier reports that the LEC HA receptor was not larger than 100 kDa (12) and was, in fact, ICAM-1 (13). ICAM-1, which is also designated in lymphocytes as CD54 (14), is not a likely candidate for the very active endocytic HA receptor of LECs because ICAM-1 is not a recycling receptor that operates via the coated pit pathway (14, 35,36). Although this identification of the LEC HAR as ICAM-1 is now recognized to be incorrect and was partially withdrawn (15), erroneous studies based on this report were published (37,38) and may be widely cited. The present results verify that ICAM-1 is not the LEC HAR because the ~90 kDa ICAM-1 is substantially smaller than the *bone fide* LEC HAR proteins purified here. We believe there are several possible reasons for this misidentification.

A 90-100 kDa protein, presumably on the surface of LECs, was initially identified as a putative HA receptor (12). However, hepatocytes and other cells have an 85-90 kDa HA-binding protein (39-41) and LECs HA-binding proteins distinct from the HAR (42) that could have also been copurified. Since their starting material was whole liver, it is likely that Forsberg and Gustafson (12) isolated a hepatocyte hyaladherin (1,2), rather than the LEC HA receptor. Without the aid of a basic assay for HA receptor activity in their preparations (12,13), they purified a 90 kDa protein from whole liver using HA-Sepharose affinity-chromatography as the final purification step (13). This molecule was then identified as ICAM-1 based on partial amino acid sequence. Subsequently this group reported that ICAM-1 was not purified on the basis of a specific interaction with HA, but rather a nonspecific interaction with the affinity chromatography matrix and linker arm (15). Therefore, ICAM-1 may only be a very weak HA binding protein. We did not detect a 90 kDa polypeptide in LECs with the properties of an HA receptor in the previous crosslinking studies (16) or in the ligand blotting experiments reported earlier (17, 18). We now know, based on the present study, that the 300 kDa HAR complex contains a ~97 kDa subunit that is larger but nonetheless similar in size, to ICAM-1. Based on reactivity with commercially available antibodies to ICAM-1, preliminary studies support the conclusion that this subunit is not ICAM-1³.

The affinity-purified LEC HA receptor preparations are nearly homogenous and contain only two very large glycoproteins when examined by nonreducing SDS-PAGE. The 175 kDa and ~ 300 kDa species are both glycoproteins containing multiple N-linked oligosaccharides. The initial partial purification of the two HAR species by preparative SDS-PAGE shows that both proteins are very heterogeneous in size². For example, the size of active 175 kDa HAR varied from about 165 kDa to 185 kDa. Even after treatment with endoglycosidase F, both proteins still give broad bands in SDS-PAGE, although they are roughly 25-30 kDa smaller.

This result indicates that either the proteins are still heavily glycosylated, phosphorylated, and/or that the core proteins are partially degraded and heterogeneous in size. Because of their large size and the broad bands, it was difficult to identify discrete intermediates during endoglycosidase-F treatment. Initial attempts to identify O-linked oligosaccharides were negative. Additional studies will be needed to determine the extent and nature of glycosylation and the basis for the size heterogeneity of both HAR species. Nonetheless, deglycosylation with endoglycosidase F did not destroy the HA-binding activity of either the 175 kDa HAR or ~ 300 kDa HAR proteins.

The 175 kDa HAR is a single protein that likely contains many internal disulfide bonds. The large apparent size increase from 175 kDa to 185 kDa upon reduction is typical for membrane receptors with extracellular domains whose compact or tightly folded structures require intraprotein disulfide bridges. Reduction of disulfide bonds eliminates the HA-binding activity of both HAR species.

The 300 kDa HAR is not a single protein, but a complex of three subunits, present in equal molar ratios. The three sizes of the reduced subunits of the 300 kDa HAR are 260 kDa, 230 kDa and 97 kDa and these are designated, respectively, the α , β and γ subunits. The 300 HAR could be a ($\alpha\beta\gamma$) heterotrimer with these three subunits being the products of several different genes. Alternatively, the 300 HAR could be a homodimer of ~300 kDa subunits, with one subunit specifically cleaved into ~97 and ~230 kDa species. Based on the broad bands even after deglycosylation, all three subunits of the 300 kDa HAR as well as the 175 kDa HAR could be complex heterogeneous mixtures of HAR produced by proteolytic processing.

A 1:1:1 complex of the three 300 kDa subunits might be expected to migrate as a >500 kDa species in nonreducing SDS-PAGE. Although the lack of good standards above 200 kDa makes it difficult to assign relative mass, the 300 kDa HAR appears to migrate anomalously fast

(i.e. to a smaller than appropriate size position). Although the relative size of such a complex should be >300 kDa, we have noted before (18) that the 175 kDa HAR and 300 kDa HAR are very elongated, not globular, molecules. Their apparent M_r s depend greatly on the pore size of the gels. During SDS-PAGE the 175 kDa and 300 kDa HAR proteins behave like elongated rods (18). Ferguson plot analysis showed that the apparent size relative to the typical globular standards was very dependent on the pore size of the gels. Therefore, the anomalous migration of the nonreduced 300 kDa HAR may be explained if the two large α and β subunits are also very extended or rod-like in the ternary complex (Fig. 6).

The overall ratio of the four proteins in our affinity purified HAR preparations has consistently been about 2:1:1:1 (for the 175, 260, 230 & 97 kDa proteins, respectively). The scheme in Fig. 7 also illustrates the question of whether one 300 kDa HAR complex, with 97, 230 & 260 kDa subunits, is associated in a larger complex with two copies of the 175 kDa HAR.

One of the monoclonal antibodies we prepared² against the 175 kDa HAR cross-reacts with the 300 kDa HAR and completely blocks the uptake of HA by live cells. This result further substantiates that these two proteins constitute the *bone fide* LEC HA receptor. Therefore, despite any uncertainty about whether there are two HARs or only one HAR, we have succeeded for the first time in purifying the endocytic HA receptor of LECs. A major question that remains to be answered is whether the 175 kDa and 300 kDa proteins are separate, independently functioning HA receptors (iso-receptors for HA) or if they function together in a very large (i.e. >700 kDa) HA receptor complex. Since the antibody used for affinity purification recognizes both the 175 kDa HAR and 300 kDa HAR, we cannot presently use immunological methods to answer this question. Preliminary experiments with a variety of hetero- or homo bifunctional crosslinkers have failed to detect an association between the 175

kDa HAR and any of the three proteins in the 300 kDa HAR complex in LEC membranes. Using biochemical, recombinant and immunologic approaches, we will continue to investigate this question.

We propose that LECs contain the 175 kDa and 300 kDa species as two highly similar but distinct and separate isoreceptors for HA. The consistent 2:1 stoichiometry of the purified 175 HAR and 300 HAR species in LECs may reflect the tight and coordinated regulation of their expression, rather than their physical association. The reason for having two HARs may be related to the great polydispersity of HA. More than one HAR may be required to mediate effective removal from the blood of HA molecules that can vary over a mass range from 10^3 to 10^6 Da. Each HA isoreceptor may be specialized to interact with either smaller or larger HA. Ongoing studies to clone and further characterize the four purified HAR subunit proteins should enable us to elucidate their structure and determine their role in normal health and a variety of disease states.

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FOOTNOTES

1. *Abbreviations.* HA, hyaluronic acid, hyaluronate, hyaluronan; HAR, HA receptor; ICAM-1, intercellular adhesion molecule-1; LECs, liver endothelial cells; Tris, trishydroxymethylamino methane; TBS, tris-buffered saline.
2. B. Zhou, J.A. Oka and P. H. Weigel (manuscript in preparation).
3. J.A. Oka, B. Zhou and P.H. Weigel unpublished results.

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FIGURE LEGENDS

FIGURE 1. Binding of the LEC HARs by a variety of lectins. NP40 extracts of LEC membranes (~50 µg) were incubated at 4°C for 2 h with 50 µl of agarose gel suspension containing one of the indicated nine lectins: concanavalin A (ConA); *Dolichos biflorus* agglutinin (DBA); *Helix aspersa* agglutinin (HAA); *Limulus polyphemus* agglutinin (LPA); peanut agglutinin (PNA); *Ricinis communis* agglutinin-I (RCA-I); soy bean agglutinin (SBA); *Ulex europaeus* agglutinin-I (UAE-I); wheat germ agglutinin (WGA). Lane 10 contains untreated extract. The lectin-gel samples were then processed as described in Methods and the eluted proteins were analyzed by SDS-PAGE followed by either electrotransfer and ligand blotting with ¹²⁵I-HA to detect HA receptor activity (A) or staining of the gel with silver (B).

FIGURE 2. Immuno-affinity purification of the LEC HARs. HA receptors were purified from NP-40 extracts of LEC membranes, followed by sequential chromatography using RCA-I-Sepharose and then monoclonal antibody 175HAR-30 coupled to CNBr-activated Sepharose as described in Methods. Protein profiles were analyzed by SDS-PAGE and silver staining. Lane 1, run-through from the RCA-I column; lane 2, the starting NP40 extract of LEC membranes; lane 3, proteins purified from the RCA-I column; lane 4, the purified HA receptors eluted from the antibody column.

FIGURE 3. Effects of reduction and endoglycosidase-F treatment on composition of the purified 175 kDa HAR and 300 kDa HA receptors. HA receptors were immuno-affinity purified as in Fig. 3 and analyzed by SDS-PAGE before and after treatment with endoglycosidase-F and/or reduction with β-mercaptoethanol as indicated. Open and solid arrows indicate, respectively,

the positions of the untreated 175 kDa and 300 kDa HA receptors. The four nondeglycosylated bands (lane 3) generated by reduction of the purified HA receptor (arrowheads) are labeled a-d.

FIGURE 4. The 175 kDa HA receptor contains a single protein, but the 300 kDa HA receptor contains three proteins. LEC HA receptors were immunopurified as in Fig. 3, subjected to SDS-PAGE and the 175 kDa HA receptor (lanes 3 & 4) and 300 kDa HA receptor (lanes 1 & 2) bands were excised, and rerun on a 7% gel with or without reduction as indicated. The position of the nonreduced 300 kDa and 175 kDa HARs are indicated by the solid or open arrows, respectively. Three proteins of approximately 97 kDa, 230 kDa and 260 kDa (solid arrowheads a, c & d) arise from reduction of the 300 kDa HA receptor (lane 2). The 175 kDa HA receptor gives a single ~185 kDa species (open arrowhead b) after reduction (lane 3). This 185 kDa protein is not seen in the 300 kDa HA receptor. The pattern observed has been reproducible in five independent HAR purifications.

FIGURE 5. Effects of reduction and endoglycosidase-F treatment on HA-binding activity of the purified 175 kDa HAR and 300 kDa HA receptors. Purified LEC HAR was reduced and/ or endoglycosidase-F treated as in Fig. 4 and assayed after SDS-PAGE by ligand blotting with ^{125}I -HA. Both the 175 kDa (open arrow) and the 300 kDa (solid arrow) HAR species are smaller after deglycosylation by 25-30 kDa but still active (lanes 1 & 2), whereas after reduction neither HA receptor is able to bind HA (lanes 3 & 4).

FIGURE 6. Model for the LEC HA receptors. The scheme summarizes the organization and composition of the affinity purified HA receptor from rat LECs. The numbers indicate the approximate mass in kDa of each protein. HA receptor preparations may contain two independent HA isoreceptors or may be a super large complex composed of two (or three) copies of the 175 kDa protein and one copy of the 300 kDa HA receptor complex. The 300 kDa HA receptor is a heterotrimeric complex of three subunits (α , β and γ) that are disulfide-bonded. Although the model shows each of the three subunits disulfide-linked to the other two, this is not yet known.

TABLE I

Affinity purified LEC HA receptor preparations were analyzed by nonreducing SDS-PAGE followed by staining with either Coomassie Brilliant blue or silver as described in Methods. Stained 175 kDa and 300 kDa protein bands were quantitated using a Molecular Dynamics Personal Densitometer. The total staining intensity for both bands was set at 1.0 and the relative amount in the 175 kDa band is shown. Based on their relative nonreduced masses determined by SDS-PAGE, the observed mole ratio of the 175 kDa and 300 kDa proteins is approximately 1.7:1.0. Using the relative masses of the three subunits of the 300 kDa HA receptor, as determined in Fig. 5, the mole ratio for the 175/300 species could be as high as 3.2 to 1.0.

Experiment	Coomassie	Silver
(fraction present in 175 kDa band)		
1	0.50	0.61
2	0.57	0.45
3	0.44	0.60
4	0.45	-
5	0.40	-
Mean:	0.47 ± 0.07	0.55 ± 0.09
0.50 ± 0.08		

TABLE II

Affinity purified LEC HA receptor preparations were first reduced with 5% β -mercaptoethanol and then analyzed by SDS-PAGE followed by staining with silver as described in Methods. Stained protein bands were quantitated using a Molecular Dynamics Personal Densitometer. The total staining intensity for the reduced 175 kDa protein (which migrates at 185 kDa) and the three bands derived from the 300 kDa HA receptor was set at 1.0. The fraction of total staining in each of the four bands is shown.

Experiment	Protein Band			
	185 kDa	97 kDa	230 kDa	260 kDa
	(fraction of the total staining)			
1	0.43	0.14	0.24	0.19
2	0.46	0.05	0.21	0.28
3	0.51	0.10	0.24	0.15
Mean:	0.47 ± 0.04	0.10 ± 0.05	0.23 ± 0.02	0.21 ± 0.07
Mole ratio:	2.5	1.0	1.0	0.8

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DATE DEPOSITED: OCTOBER 27, 2003



Practitioner's Docket No. 5820.603

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Weigel, et al.

Application No.: 09/842,930 Group No.: 1647
Filed: April 25, 2001 Examiner: L. Spector
For: HYALURONAN RECEPTOR FOR ENDOCYTOSIS

Mail Stop - RCE
Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

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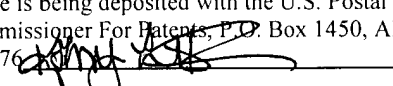
STATEMENT OF BIOLOGICAL CULTURE DEPOSIT

WARNING: *Submission of a letter related to a biological deposits after a Notice of Allowance may subject an application to a reduction in patent term adjustment under 37 C.F.R. 1.704(c)(10). See Notice of May 29, 2001, 1247 OG 111-112, June 26, 2001.*

I, Paul H. Weigel
Name of Applicant or Assignee or Assignee's representative
hereby state:

1. That the following cultures referred to in the specification of this application have been deposited:

<u>Mouse hybridoma: mAb-28</u>	<u>PTA-5354</u>
Strain	Accession Number
<u>Mouse hybridoma: mAb-30</u>	<u>PTA-5355</u>
Strain	Accession Number
<u>Mouse hybridoma: mAb-54</u>	<u>PTA-5356</u>
Strain	Accession Number
<u>Mouse hybridoma: mAb-159</u>	<u>PTA-5358</u>
Strain	Accession Number
<u>Mouse hybridoma: mAb-174</u>	<u>PTA-5359</u>
Strain	Accession Number
<u>Mouse hybridoma: mAb-235</u>	<u>PTA-5360</u>
Strain	Accession Number
<u>Mouse hybridoma: mAb-467</u>	<u>PTA-5361</u>
Strain	Accession Number

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage by Express Mail, EV 272908025 US, addressed to: Commissioner For Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on 10/27/2003. By Kathryn L. Hester, Ph.D., Reg. No. 46,764  27

2. That the date of the above deposit is:

(check appropriate item below)

- ☐ before the U.S. filing date of this application.
- ☒ after the U.S. filing date of this application and proof that the cultures identified above are the same cultures described in the application as filed is described hereinbelow. The facsimile dated 9/23/2003 from the American Type Culture Collection lists the designations for each mAb deposited therewith. Such mAb designations are the same as designations for the mAb's described in the Specification of the subject application (with the exception of a number and letter extension) thus providing proof that the cultures identified above are the same cultures described in the application as filed.

3. That the name and address of the depository is:

American Type Culture Collection
10801 University Boulevard
Manassas, VA 20110-2209

4. That a statement that the cultures deposited with the above named depository were viable and were capable of reproduction, if appropriate, on the date of deposit is attached. Such statement is executed by:

(complete a, b or c below)

- a. ☒ the depository.
- b. ☐ the applicant.
- c. ☐ a competent third party.

WARNING: *If the applicant himself or a competent third party makes the statement that the microorganism is viable and is capable of reproduction, if appropriate, then the tests must have been performed on a sample certified to have been (1) received by the depository with the same samples actually deposited and (2) promptly returned to applicant.*

5. That, with respect to the permanence of the cultures deposit:

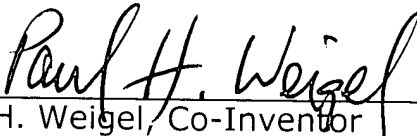
(complete a, b, or c)

- a. ☒ the depository is an official depository, in accordance with the Budapest Treaty for the above deposited cultures.
- b. ☐ the depository affords permanence of the deposit for at least 30 years or at least 5 years after the most recent storage request, whichever is longest.
- c. ☐ evidence that permanent availability of the microorganism is assured is provided in the form of the attached copy of the contract with the above-mentioned depository with respect to the deposited cultures.

I state that should the microorganisms mutate, become nonviable or be inadvertently destroyed, applicants will replace such microorganisms for at least 30 years from the date of the original deposit, or at least 5 years from the date of the most recent request for release of a sample or for the life of any patent issued on the above-mentioned application, whichever period is longer.

6. That, with respect to availability of the cultures, I state that the deposit has been made under conditions of assurance of (a) ready accessibility thereto by the public if a patent is granted whereby all restrictions to the availability to the public of the culture so deposited will be irrevocably removed upon the granting of the patent (M.P.E.P. § 608.01 (p)), and (b) access to the culture will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 C.F.R. § 1.14 and 35 U.S.C. § 122.

- ☐ Evidence of the accessibility of the cultures as set forth above is provided in the form of the attached copy of the contract with the above mentioned depository with respect to the deposited cultures.



Paul H. Weigel, Co-Inventor

10-24-03
Date

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.

To: (Name and Address of Depositor or Attorney)

University of Oklahoma
Dept of Biochemistry and Molecular Biology
Attn: Paul H. Weigel
BMSB Rm 860
PO BOX 26901
Oklahoma City, OK 73190

Deposited on Behalf of: The Board of Regents for the University of Oklahoma

Identification Reference by Depositor:

Patent Deposit Designation

Mouse Hybridoma: mAb-28.1C	PTA-5354
Mouse Hybridoma: mAb-30.1A	PTA-5355
Mouse Hybridoma: mAb-54.1E	PTA-5356
Mouse Hybridoma: mAb-159.1B	PTA-5358
Mouse Hybridoma: mAb-174.1F	PTA-5359
Mouse Hybridoma: mAb-235.1B	PTA-5360
Mouse Hybridoma: mAb-467.1C	PTA-5361

The deposits were accompanied by: a scientific description a proposed taxonomic description indicated above. The deposits were received July 30, 2003 by this International Depository Authority and have been accepted.

AT YOUR REQUEST: X We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested August 11, 2003. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:

Marie Harris
Marie Harris, Patent Specialist, ATCC Patent Depository

Date: September 29, 2003

cc: Douglas Sorocco, Esq.
Ref: Docket or Case No.: 5820.603